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Research paper

Intracellular antibodies for proteomics[☆]

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Abstract

The intracellular antibody technology has many applications for proteomics studies.

The potential of intracellular antibodies for the systematic study of the proteome has been made possible by the development of new experimental strategies that allow the selection of antibodies under conditions of intracellular expression. The Intracellular Antibody Capture Technology (IACT) is an *in vivo* two-hybrid-based method originally developed for the selection of antibodies readily folded for ectopic expression. IACT has been used for the rapid and effective identification of novel antigen–antibody pairs in intracellular compartments and for the *in vivo* identification of epitopes recognized by selected intracellular antibodies. IACT opens the way to the use of intracellular antibody technology for large-scale applications in proteomics. In its present format, its use is however somewhat limited by the need of a preselection of the input phage antibody libraries on protein antigens or by the construction of an antibody library from mice immunized against the target protein(s), to provide an enriched input library to compensate for the suboptimal efficiency of transformation of the yeast cells. These enrichment steps require expressing the corresponding proteins, which represents a severe bottleneck for the scaling up of the technology.

We describe here the construction of a single pot library of intracellular antibodies (SPLINT), a naïve library of scFv fragments expressed directly in the yeast cytoplasm in a format such that antigen-specific intrabodies can be isolated directly from gene sequences, with no manipulation whatsoever of the corresponding proteins. We describe also the isolation from SPLINT of a panel of intrabodies against a number of different proteins.

The application of SPLINT on a genome-wide scale should help the systematic study of the functional organization of cell proteome.

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1. Introduction

The explosive growth in the amount of data coming from current high-throughput genomic and proteomic methodologies calls for the development of novel tools to study protein functions and protein–protein interactions.

[☆] From genes to intracellular antibodies: SPLINT, a single pot library for the selection of functional intrabodies.

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One of the fundamental problem in functional genomics is to find a general technique to interfere with protein–protein and protein–DNA interconnections in complex regulatory and signalling networks.

Intracellular antibodies (Biocca et al., 1990) have been shown to be a valuable tool for studying numerous aspects of biological processes, opening up new experimental opportunities to analyze the function of a wide range of cellular molecules (Biocca and Cattaneo, 1995; Richardson and Marasco, 1995; Cattaneo and Biocca, 1997).

Intracellular antibodies, or intrabodies, not only exhibit highly specific molecular recognition properties but are also able to modulate the function of their cognate targets in a highly specific manner. Antibodies expressed inside cells have already demonstrated their impressive potential as intracellular inhibitors (Cochet et al., 1998; Biocca et al., 1993; Biocca and Cattaneo, 1995; Gargano et al., 1996; Levy-Mintz et al., 1996; Mhashilkar et al., 1995).

The intrabody concept is based on a phenotypic knockout of intracellular proteins and exploits the specific targeting of antibodies to subcellular compartments. As intrabodies are derived from the virtually unlimited repertoire of antibodies, there are basically no limitations on the choice of target molecule. Other strategies for the inactivation of specific intracellular proteins are being developed. Among these, RNA interference (RNAi) is generating growing interest. The RNAi approach, however, is limited by incomplete RNA cleavage, inaccessible RNA sequences and proteins with a long half-life. In addition, RNAi cannot be used to target specifically individual protein–protein interactions or post-translational modifications of proteins. With intracellular antibodies, it is possible to develop a reagent that prevents particular associations but spares others. Last but not least, once an intrabody has been developed, able to inhibit a cellular function, it provides a direct biochemical handle on the recognized protein and its binding partners. Intrabodies have therefore a great added value for functional proteomics and the study of protein–protein interactions.

Notwithstanding its success and its potential, a bottleneck of this protein knockout technology became evident (Biocca et al., 1990; Cattaneo and Biocca, 1999), in that not all antibodies present in

natural or synthetic antibody repertoires appear to perform well when expressed intracellularly, due to what was called the “disulphide bond problem” (Biocca et al., 1995). Moreover, it became clear that being a “good intracellular antibody” requires the concomitant optimization of a complex list of properties, including stability, in vivo half-life, solubility, propensity to aggregate, affinity and specificity (Cattaneo and Biocca, 1999). A “good intracellular antibody” is defined as an antibody that is able to fold tolerating the conditions found in the cell cytoplasm and to have an affinity high enough to interact effectively with its target antigen. Rather than attempting to rationally engineer antibodies with these properties, we showed recently that in vivo selection processes can be applied to rapidly and effectively select intrabodies against several targets (Visintin et al., 1999, 2002; Tse et al., 2002). This yeast two-hybrid-based technology named Intracellular Antibody Capture Technology (IACT) (Visintin et al., 2002) proved to be a valuable method for the selection of intracellular antibodies on the basis of their ability to bind antigen intracellularly. Most importantly, we and others showed that the selection of an antibody by IACT is a good and reliable predictor for being a reliable intrabody in mammalian cells (Visintin et al., 1999, 2002; Tse et al., 2002).

The development of IACT may therefore overcome the severe bottleneck that limited the exploitation of intracellular antibodies as the technology of choice for large-scale protein knockout studies in cells, and therefore for functional proteomics. It is therefore very important to further optimize the selection procedure to isolate validated intracellular antibodies and to remove residual limitations to the present selection technologies. In its present implementation, IACT uses sources of antibody variable regions genes that have been independently generated (Fig. 1). In these repertoires, “good intracellular antibodies” are a subset of the total antibody repertoire. The large size of these libraries, together with the limited efficiency of DNA transformation in yeast cells (required for the intracellular selection step), requires necessarily some preselection or enrichment step, in order to reduce the size of the input library to a complexity compatible with its downstream expression in yeast cells. Indeed when making intracellular antibody libraries for IACT selection, V genes might

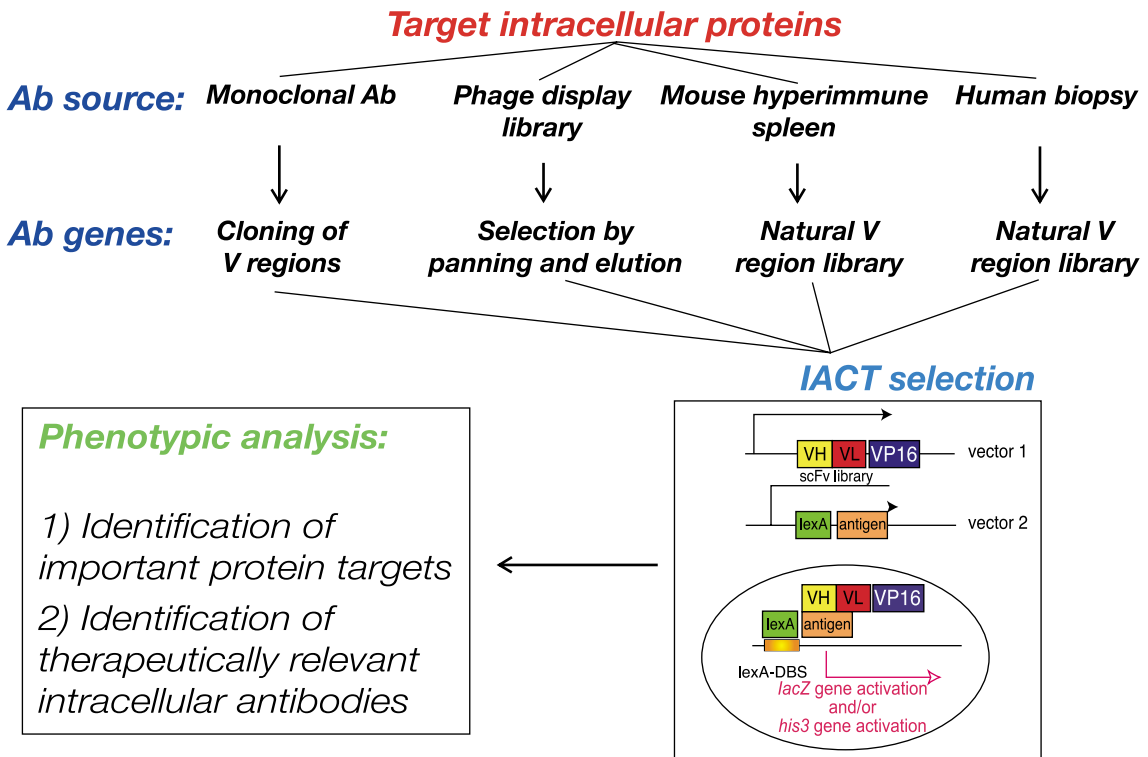


Fig. 1. Diagram of the construction of a SPLINT library. The antibody variable regions can be cloned directly from a non-immunized mouse on a vector that expresses the activation domain and two NLS fused in tandem after the scFv antibody fragment. The SPLINT library is then inserted in the yeast strain in an episomal form and can then be used for IACT screening immediately after transformation of the antigen to be used as bait.

be derived from lymphocytes which may have undergone antigen stimulation (Vascotto et al., submitted for publication) or from the results of a phage antibody library selection (Visintin et al., 2002). In the former case, antigen immunization represent the enrichment step (Vascotto et al., submitted for publication) while in the latter case one cycle of in vitro panning on antigen columns is required (Visintin et al., 2002). High-throughput “from gene to intrabody” approaches would require immunization or preselection to be bypassed.

A solution of this “impedance mismatch” limitation would be to create libraries that are enriched at the source in “good intracellular antibodies”. One way to do so would be to exploit the intracellular consensus sequences (ICS) identified from sequences of antibodies selected with IACT (Visintin et al., 2002), but this requires the construction of ad hoc engineering or ad hoc libraries (Tanaka et al., 2003)

and poses concerns as to whether this will provide the necessary diversity.

We explored in this paper the possibility of exploiting naïve repertoires of natural V regions, superseding the need for framework consensus engineering. In planning the construction of an intracellular antibody library, the important parameters to consider are diversity and affinity range, and the relationship existing between these parameters.

Antibody libraries exist of a complexity comparable to the natural immune system of mammals, so that theoretically their repertoire is complete and every imaginable antibody may be derived. In general, the larger than the library, the higher the chance is to isolate specific antibodies with good binding characteristics (in terms of affinity) (Perelson and Oster, 1979; Griffiths et al., 1994). It has been shown that libraries containing about 10^7 independent clones yielded antibodies to proteins with affinities in the low micromolar

range, similar to those obtained in a primary immune response in vivo, while larger antibody libraries of 10^9 – 10^{10} clones yielded specific antibodies with affinities in the nanomolar range, similar to those obtained after a secondary immune response (Griffiths et al., 1994; Vaughan et al., 1996; Sheets et al., 1998). It is also obvious that the real diversity of a library must take into account the number of correctly assembled scFv, the number of frame shift, deletions or stop codons present in the V gene sequences and other quality related factors (Knappik et al., 2000). Therefore the important parameter is the diversity of *functional* antibodies in the library, rather than the *nominal* diversity of the library. This parameter must be evaluated for all the libraries constructed. Indeed non-functional antibody fragments drastically lower the true size of the library, normally evaluated by counting the number of colonies after DNA transformation.

As for the affinity range required for an intrabody library, a reference value is provided by the interaction affinity between proteins involved in eukaryotic signal transduction pathways. For example, the affinity of Pro-rich peptide sequences for the SH3 domain is relatively weak (5–100 μ M) (Dalgarno et al., 1997; Zucconi et al., 2001), suggesting that an intracellular inhibitor would not need a very high affinity to compete with the ligand–SH3 interaction motifs. We demonstrated that intracellular antibodies with affinities in the range of 100 nM could effectively divert their target antigens from their natural intracellular environments thereby inhibiting it (Visintin et al., 2002). In general, intracellular antibodies with affinity in the micromolar/nanomolar range have been shown to be effective intracellular inhibitors (Cattaneo and Biocca, 1997). In fact, choosing an antibody based on a higher affinity in vitro alone is a poor predictor of its function as an intrabody in vivo and half-life and stability of intracellular antibodies have been shown to be more relevant for good intracellular inhibition than affinity per se (Zhu et al., 1999), (Rajpal and Turi, 2001; Arafat et al., 2000). In addition, the precise subcellular targeting of antibodies increases the local concentration of the two interactors, compensating for lower affinity.

Finally, one could expect that antibody domains that aggregate or fold poorly when expressed intracellularly would provide a growth disadvantage for the cells that express them (Cardinale et al., 2003).

This would provide a baseline antigen-independent selection for “good intrabodies”, on which antigen-dependent selection schemes could be superimposed (Visintin et al., 2002; Tse et al., 2002).

Altogether, these considerations suggested the possibility that a naïve library, with a size compatible with the efficiency of transformation of yeast cells, should be large enough to select specific intracellular antibodies for in vivo knockout studies. Here we describe the design, construction, analysis and selection of a single pot library of intrabodies (SPLINT), and describe its applications to accelerate proteomics programs.

2. Results

In a parallel study (Vascotto et al., submitted for publication), a library of V regions, derived from hyperimmune spleens from mice immunized with the rotavirus NSP5 protein, was created to select intracellular antibodies by IACT. The selection and the characterization of this library gave unexpected results:

- (i) Many antibodies against the NSP5 were isolated, and the sequences of anti-NSP5 antibody V regions obtained from the NSP5 antigen selections were often very similar to germline sequences (for VH or VK).
- (ii) The antibody library obtained from NSP5 immunized splenocytes was also found to contain many antibodies against different antigens, against which no immunization had been made.
- (iii) The selected intrabodies were found not to be promiscuous and to have high specificity.

Moreover, the IAC technology has been shown previously, in one anecdotal example, to lead to the isolation of putative intracellular binders starting directly from a small naïve antibody library cloned in the two-hybrid vector (Portner-Taliana et al., 2000), with no library preselection. The authors showed that using IAC technology it was possible to select one specific in vivo binder against their target antigen although no functional characterization of the selected intracellular antibody was provided.

On the basis of these results, as well as on the basis of the considerations made in the previous section, we explored the possibility that a small but systematically very well-examined naive library of antibodies directly cloned in the two-hybrid vector could yield a good single pot library of intracellular binders.

2.1. Construction of scFv repertoire

The cloning scheme used to create the scFv library is outlined in Fig. 2. Natural V regions were cloned from total RNA extracted from lymphocytes pooled from three mouse spleens.

After cDNA synthesis performed by random hexamer priming, V regions were amplified by using a set of partially degenerated 5' and 3' primers (Orlandi et al., 1992), suitably re-designed (Sblattero and Bradbury, 2000). V regions from cDNA were also re-amplified, to add extra restriction sites for their assembly into scFv fragments. VH and VL gene mix were assembled together in the scFv format by overlap extension PCR (Sheets et al., 1998). The scFv assembled library was then subcloned for expression as a VP16 fusion in pLinker220 plasmid (Fig. 3). The bulk DNA for the scFv-VP16 library extracted from $\sim 10^7$ clones was then transformed in L40 yeast cells to generate a naïve population of scFvs-VP16 to be used readily for the selection of intracellular antibodies against different antigens, by using in vivo two-hybrid system. Cloning efficiency close to 100% was confirmed by PCR amplification of randomly isolated bacterial and yeast clones (data not shown).

One hundred independent antibodies were chosen at random and analyzed by sequencing and by separately amplifying VH and VL genes and fingerprinting them with the restriction enzyme *Bst*NI. All the analyzed clones display a different sequence and fingerprinting pattern, confirming the diversity of the library. An examination of the deduced amino acid sequences of randomly picked clones showed that they were all different and comprised a variety of different subgroups and germline sequences (data not shown).

2.2. Selection of scFv intracellular antibodies with different specificities by IACT

To verify whether we could select from this library intracellular antibodies against signalling proteins,

and other intracellular proteins, we screened the library against a panel of different baits (Table 1). These include, two domains of Shc, K-RAS, Syk, β 4-thymosin ubiquitous, β 4-thymosin lymphoid, amyloid β 1–42, gephyrin, TFII-I and X5 ORF SARS-CoV proteins.

The baits chosen for the validation of the SPLINT library include proteins of different species, of viral origin and of very different function and cellular location:

- Two different domains (CH2 and SH2) of human p66^{Shc} adaptor protein (Ventura et al., 2002), which contains a unique amino-terminal proline-rich region (CH2) an NH2-terminal phosphotyrosine binding domain (PTB) followed by a collagen homology domain (CH1) and a COOH-terminal SH2 domain. By virtue of its PTB and SH2 domains, Shc proteins link receptor and non-receptor tyrosine kinase activation to downstream cytoplasmic effectors and can bind to activated receptors that have been tyrosine-phosphorylated. This protein is implicated in pathways activated by environmental stress and regulation of life-span.
- Human K-Ras is a monomeric membrane-localized small 21 kDa G protein that functions as a molecular switch for cytoplasm to nuclear signalling (Ehrhardt et al., 2002). The ras-signalling pathway is an attractive target for anticancer therapy because of its important role in carcinogenesis.
- Syk protein-tyrosine kinase has been implicated in a variety of haematopoietic cell responses, in particular immunoreceptor signalling events that mediate diverse cellular responses including proliferation, differentiation, and phagocytosis. Syk appears also to play a general physiological function in a wide variety of cells including neuronal cells in which it is suggested to play an important role in signalling steps to neurite extension (Yanagi et al., 2001).
- Thymosin β 4 encodes both an ubiquitous actin-binding protein (UT β 4) with demonstrated capacity to inhibit neutrophilic infiltration, and a splice-variant limited to lymphoid tissue (LT β 4) that was shown to be expressed in activated dendritic epidermal T cells, and other intraepithelial lymphocytes, to down-regulate local inflammation (Girardi et al., 2003).

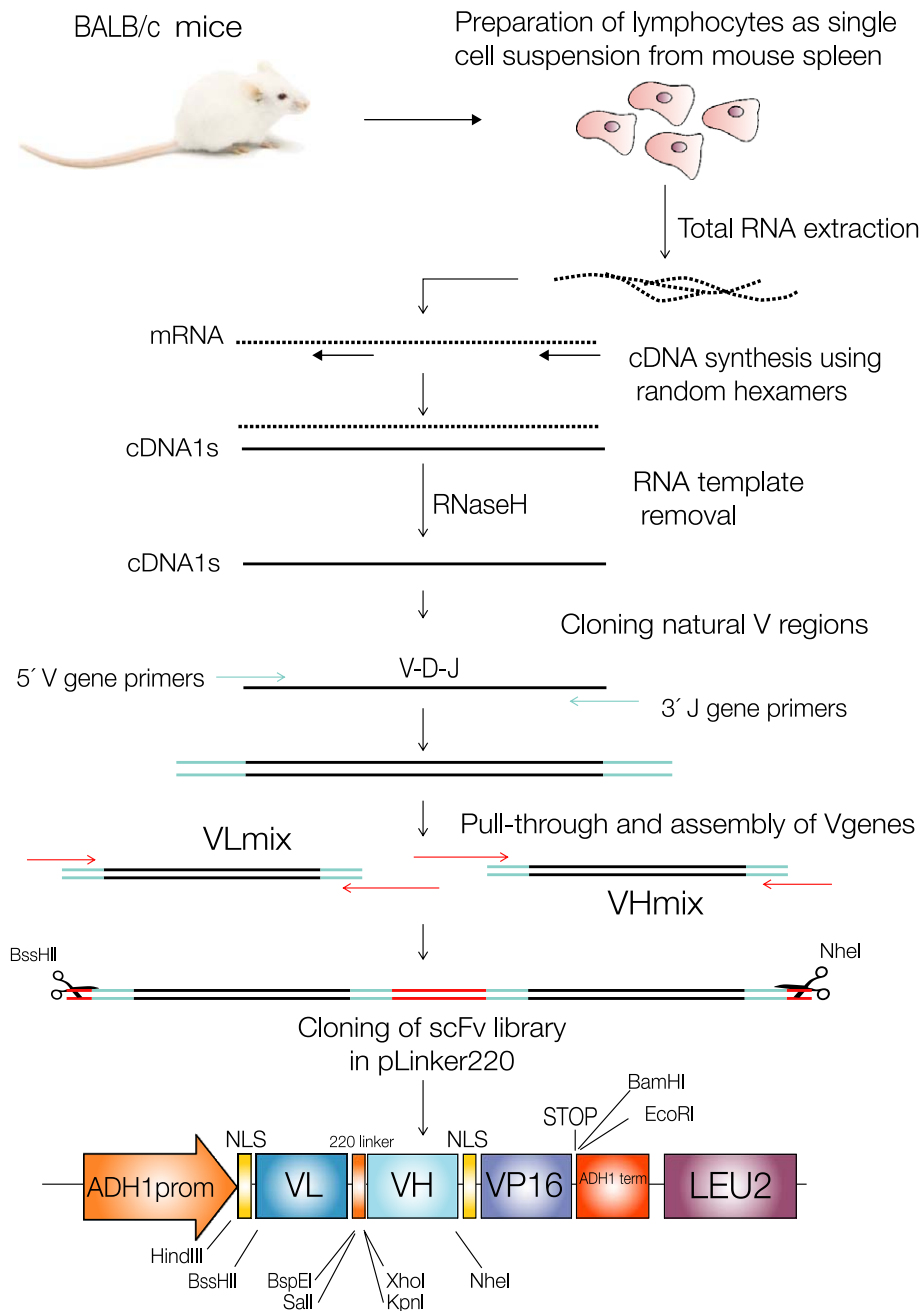


Fig. 2. Construction of SPLINT. Mouse V genes were amplified with degenerated primers from total cDNA. A second PCR generated products with common 5' and 3' ~ 30-nucleotide tails. The common 3' ~ 30-nucleotide ends allow assembly of full scFv genes and the common 5' ends allow the cloning into linearized pLinker220 vectors into *BssHIII* and *NheI* restriction sites.

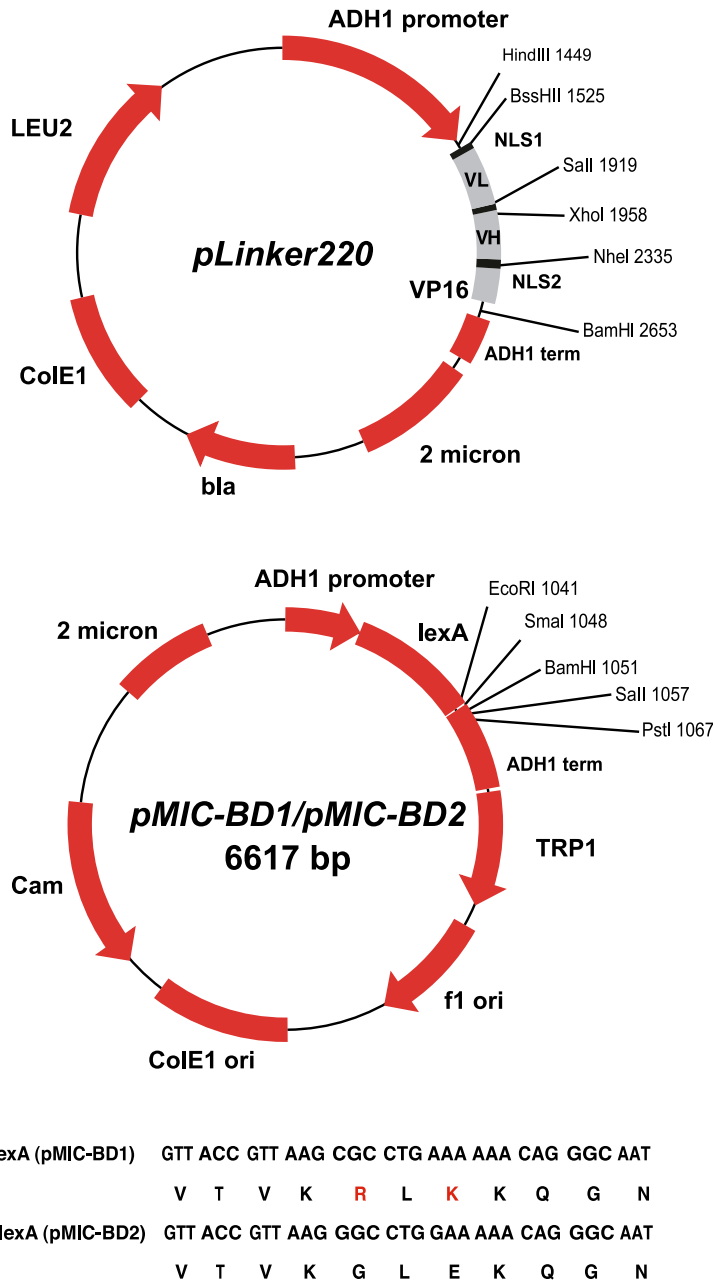


Fig. 3. SPLINT vectors. pLinker220 vector used for expression of SPLINT library as fusion to the VP16 activation domain. The LEU2 gene permits maintenance of the plasmid and selection on media lacking leucine in yeast strain L40. The bla gene permits the selection of plasmid in *E.coli*. pMIC-BD 1 and 2 vectors are derivative of pBTM116. They use the strong alcohol dehydrogenase promoter (ADH1) to express bait proteins as fusions to the DNA-binding protein lexA. The plasmids contain the TRP1 selectable marker and the 2 μ m origin of replication to allow propagation in yeast, and the chloramphenicol resistance gene (Cam) to allow the selection in *E. coli*. Codons CGC and AAA of lexA within pMIC-BD1 were mutated to GGC and GAA, respectively, generating mutlexA and converting pMIC-BD1 in pMIC-BD2.

Table 1
Panel of baits used for SPLINT selection

(1) Human p66Shc CH2 domain (CH2-Shc)
(2) Human p66Shc SH2 domain (SH2-Shc)
(3) Human K-RAS
(4) Human Syk
(5) Mouse β 4-thymosin ubiquitous (TU β 4)
(6) Mouse β 4-thymosin lymphoid (LT β 4)
(7) Human amyloid β 1–42
(8) Rat gephyrin
(9) Human TFII-I
(10) X2, X5 ORF SARS-CoV

- Gephyrin is a 93-kDa protein that copurified with the glycine receptor (GlyR,) and was found to be localized at the postsynaptic side of glycinergic synapses (Prior et al., 1992; Sassoe-Pognetto and Fritschy, 2000; Craig and Lichtman, 2001).
- beta amyloid (A β) is the major constituent of senile plaques in Alzheimer's disease, a 42-aa peptide generated from a family of differentially spliced, type-1 transmembrane domain (TM)-containing proteins, called APP, by endoproteolytic processing. Amyloid β peptide (1–42) may be central to the pathogenesis of AD (Sinha and Lieberburg, 1999).
- TFII-I is an inducible multifunctional transcription factor that is activated in response to a variety of extracellular signals and translocates to the nucleus to turn on signal-induced genes (Roy, 2001).
- Severe acute respiratory syndrome (SARS) is a life-threatening form of atypical pneumonia. SARS-CoV genome is ~ 29.7 kb long and contains 14 open reading frames (ORFs) flanked by 5' and 3' untranslated regions. Coronaviruses encode a number of non-structural proteins. These non-structural proteins, which vary widely among the different coronavirus species, are of unknown function and seem to be dispensable for virus replication (Rota et al., 2003; Snijder et al., 2003; Zeng et al., 2003).

DNA sequences coding for each of these proteins, fused to *lexA* protein to create antigen-specific baits, were introduced by transformation into yeast cells expressing the SPLINT library and IACT selection was performed as described (Visintin et al., 2002). It should be noted that no manipulation whatsoever of the corresponding proteins was performed.

Double transformants were selected for histidine prototrophy and *lacZ* activity. In the search for antigen-binding scFv fragments, about 10–20 His⁺/*lacZ*⁺ different clones for each antigen were analyzed in a secondary two-hybrid screening, except for UT β 4, LT β 4, A β 1–42, gephyrin and TFII-I (Table 2) where secondary screening was more exhaustive (more than 90 His⁺/*lacZ*⁺ different clones). Specifically binding clones were obtained in each case, demonstrating that bait-specific single-chain antibodies could be expressed in the cytoplasm and in the nucleus of yeast cells (Fig. 4; Table 2). Subsequent secondary screening confirmed that true positives could be identified that interact specifically with the original bait, but not with other *lexA* fusions (*lexA*-lamin).

Eight clones for CH2-Shc, 5 clones for SH2-Shc, 2 for K-RAS, 6 for Syk, 11 for LT β 4, 2 for UT β 4 and 6 for gephyrin, 6 for A β 1–42, 4 for TFII-I and 6 for X5 ORF SARS-CoV were identified that interact with their antigens but did not interact with the unrelated *lexA*-lamin bait (Fig. 5).

A subset of antibody fragments obtained from LT β 4 screening were also analyzed for binding to UT β 4.

The lymphoid-specific thymosin encodes for a six-residue NH2-terminal extension relative to the

Table 2
Selection of ICABs with different antigen specificities

Antigen	cfu/ μ g SPLINT- DNA in L40	No. of His3 ⁺ - <i>lacZ</i> ⁺ rescued clones	No. of His3 ⁺ - <i>lacZ</i> ⁺ analyzed clones	No. of positive clones
CH2-Shc	2.4×10^6	430	20	8
SH2-Shc	3.7×10^6	189	20	5
K-RAS	4.4×10^6	27	27	2
Syk	1.4×10^6	3000	10	6
UT β 4	3×10^7	300	96	3
LT β 4	2.1×10^6	123	123	13
Gephyrin	2.6×10^6	5824	50	5
TFII-I	1.29×10^6	1000	96	4
A β 1–42	1.2×10^6	900	66	6
ORF X5 SARS- CoV	6.8×10^5	34	34	6

Summary of different SPLINT selections. The efficiency of transformation, the number of scored clones and the number of positive and validated scFv obtained are shown.

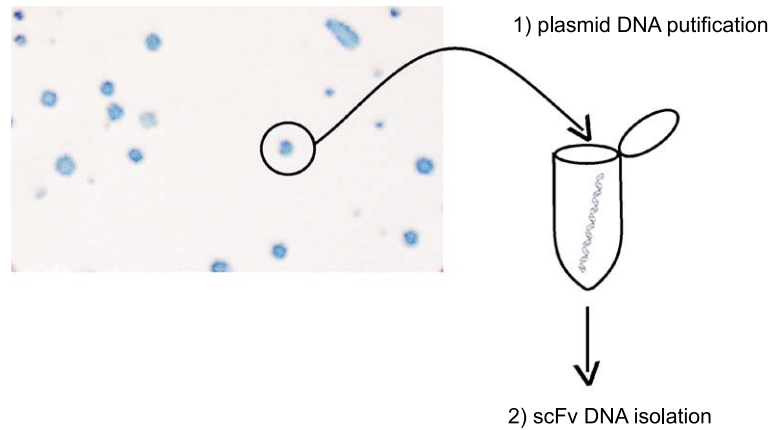


Fig. 4. Selection of specific interacting scFv–Ag pairs from primary transformation pool. After transformation of SPLINT library in antigen containing yeast strain, cotransformed library is plated to medium selective for activation of reporter genes. Colonies arising on selective plates were tested for activation of HIS3 and lacZ genes. Positive clones (His^{3+} and lacZ^{+}) were used for PCR amplification with scFv-based primers, and amplified products were used for *Bst*NI fingerprinting analysis.

ubiquitous form of thymosin. In order to verify whether it would be possible to isolate scFv specific only for the lymphoid form, we chose from the LT β 4 screening five antibody fragments that were shown to have the most different amino acid sequences among the scFv isolated, and tested them for their ability to bind UT β 4. All the scFvs tested result

to be highly specific for the lymphoid form and failed to bind the ubiquitous one (Fig. 6). The parallel selection of SPLINT on UT β 4 resulted to be very stringent; very few different fingerprinting patterns were found among 96 analyzed clones. Moreover, it was shown that the selection of SPLINT against the UT β 4 bait was able to isolate

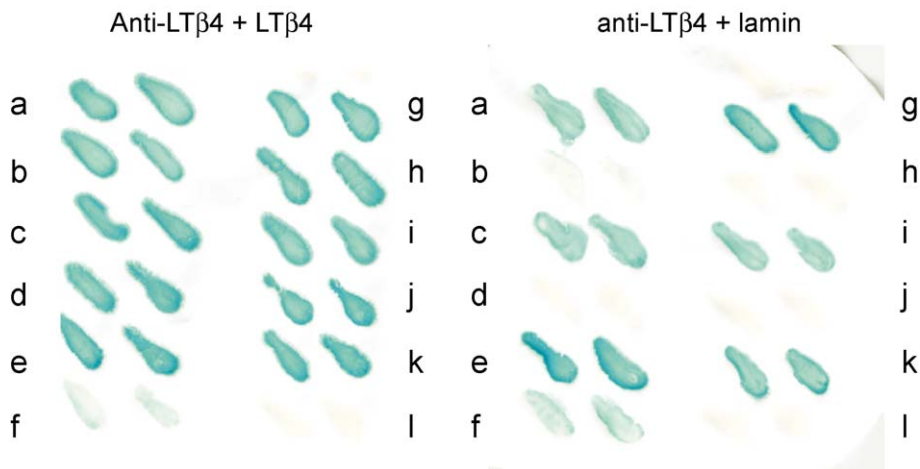


Fig. 5. Rescue scFv plasmids are reintroduced into original selection strain and into different bait strains to verify specificity. This step is very important to confirm the interactions obtained in the primary selection strategy and to confirm the specificity of antibody–target antigen interactions. The isolated plasmids expressing the scFvs (i.e. anti-LT β 4) are reintroduced into strains containing target antigen (LT β 4) and another unrelated antigen (lamin). As an example, clones b and h result to be specific only for LT β 4 and fail to interact with lamin antigen, thus confirming their specificity for LT β 4.

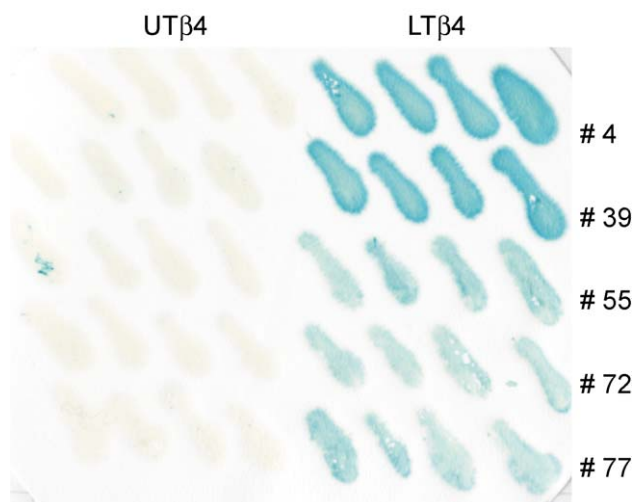


Fig. 6. Anti-LT β 4 scFv fragments specifically recognized LT β 4 and fail to recognize the UT β 4 bait. The thymosins are a family of small proteins entirely composed of a single WH2 domain, a \sim 35-residue actin monomer-binding motif, which is found in many different regulators of the actin cytoskeleton. The lymphoid-specific thymosin encodes for an extra-six-residue NH₂-terminal extension. Five antibody fragments obtained from the LT β 4 screening were tested for their ability to bind UT β 4. As shown in the figure, all the scFvs tested result to be specific only for the lymphoid form and not for the ubiquitous one.

scFvs whose amino acid sequences correspond to some scFvs previously isolated against LT β 4, thus indicating that the selection of SPLINT against LT β 4 was exhaustive enough for fishing out specific antibodies against both forms of the thymosin protein.

2.3. Improving the stringency of selections

The results summarized in Table 2 shows that for each target protein chosen, a number of different antigen-specific antibodies could be isolated directly from gene sequences. The procedure has been experimentally optimized and refined and is very robust, rapid and efficient. In some situations, it may be useful to push the selection conditions further, to fish out very strong binders and to improve the stringency of selections. To this aim, we engineered a modified LexA bait: the bacterial LexA protein of pMIC-BD1 vector was modified (mLexA) to abolish its intrinsic NLS, as previously described (Rhee et al., 2000) (Fig. 3). In this modified format, the NLS is provided only by the antibody “prey” vector. To verify whether it would be possible to screen SPLINT also with antigen expressed only in the yeast cytoplasm (as in the case with the pMIC-BD2 vector), we chose the protein K-RAS as target antigen. The choice of RAS was also

due to the apparent difficulty of isolating intracellular antibodies in the standard selection procedure (Table 2). In this modified selection procedure, the interaction between the antigen and the scFv must occur in the cytoplasm, because the antigen is expressed in yeast cells without the intrinsic nuclear localization signal of lexA. ScFv and antigen interact in the cell cytoplasm before the complex is translocated into the nucleus and activates transcription (Fig. 7). We tried in parallel the selection of anti K-RAS antibodies as lexA-bait fusion protein with or without nuclear localization signal. In both cases, we obtained the same number of functional anti K-RAS intracellular antibodies.

This indicates that the technology, in its modified format, allowed the selection of scFv with an affinity high enough to bring the antibody–antigen complex into the nucleus and to activate the transcription of reporter genes.

As a general comment, the selections with K-RAS, either with or without NLS, gave very few positive clones if compared with other antigens (Table 2). It should be noted that very few positive clones were also obtained in other reported IACT selection strategies when RAS was used as a bait (Tanaka and Rabbitts, 2003). This might indicate that the antigen has diffi-

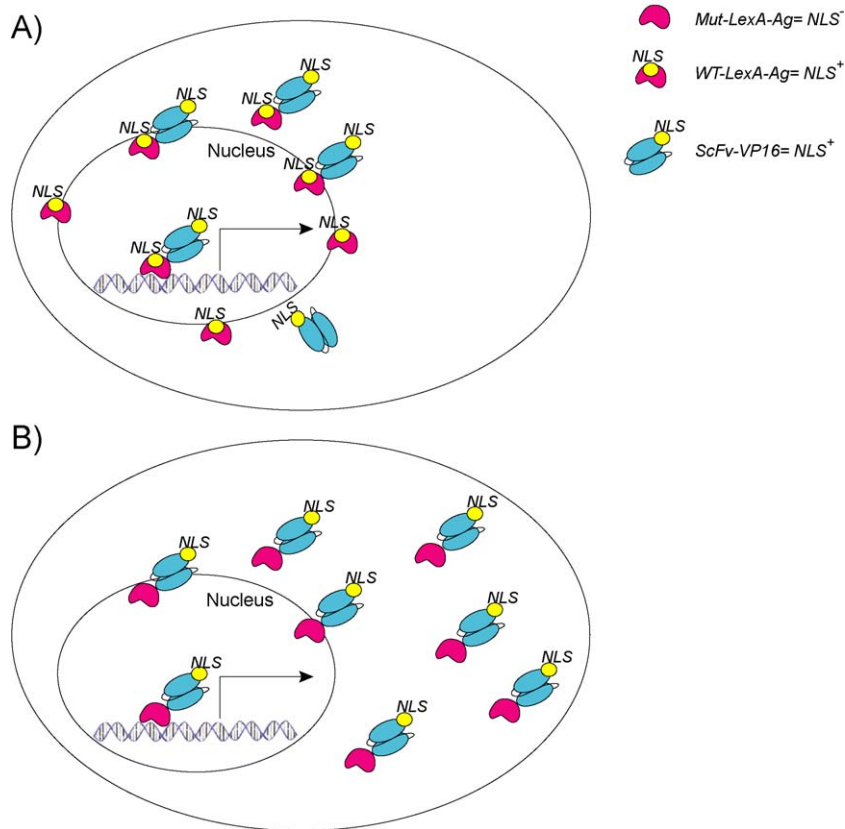


Fig. 7. Improved stringency of SPLINT selections. (A) In the original version of IACT, the interaction of an antigen with the corresponding antibody fragment could occur in both cytoplasm and nucleus because either the lexA DNA-binding domain or the scFv fragments have a SV40 large T-antigen nuclear localization signal (NLS). (B) The bacterial lexA protein was modified (mut-lexA) to abolish its intrinsic NLS and fused to the target antigen with or without the NLS. In this strategy, if a tested protein fused to mut-lexA is recognized by a scFv in the cytoplasm, the complex Ag–scFv will enter the cell nucleus and activate the reporter gene expression.

culty entering into the nucleus, and this difficulty interferes with the normal selection procedure. Alternatively, interference with the endogenous RAS protein, by the selected antibodies, possibly due to the expected anti proliferative effect of the anti RAS antibodies, might explain this low number of colonies obtained from this selection. In any case, this did not prevent the successful isolation of functional binders, even in this “difficult” case.

As discussed above, passing the IAC selection funnel is a strong and reliable predictor for the in vivo performance of an antibody. Indeed IACT selection predicts the property of being a functional intrabody in all cases (Visintin et al., 2002; Tse et al., 2002). In fact, this functional intrabody screen is a

much better predictor than in vitro scFv characterization. In any case, in order to provide further additional support to the validation of the SPLINT library described here, the binding properties of antibodies selected from SPLINT were also confirmed in vitro by ELISA. One significant example for one such antigen–antibody group is reported for anti-amyloid β 1–42 antibodies (Fig. 8). This characterization was extended, for a number of the ICABs selected from SPLINT, to their in vivo performance when expressed in mammalian cells, where they showed good solubility and half-life (data not shown).

In conclusion, we established that functional antigen-specific ICABs were selected from the natural

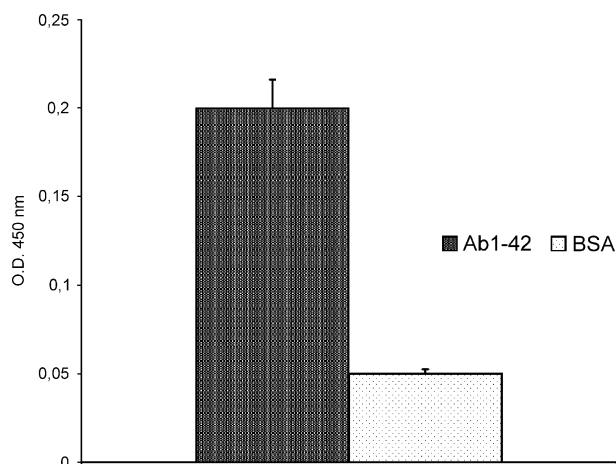


Fig. 8. In vitro binding specificity of isolated intrabodies. ELISA of soluble fractions of *E. coli* cultures expressing one randomly chosen anti- $\text{A}\beta$ 1–42 in the periplasmic space. Antigens were coated at 10 $\mu\text{g}/\text{ml}$. The ELISA signals were measured at O.D. 450.

SPLINT library against a wide spectrum of very different protein antigens, including viral proteins.

3. Discussion

We describe an evolution of the IACT genetic selection method that allows the isolation of functional intrabodies recognizing gene products directly from the sequences of those gene products, without the need to produce the proteins.

The major goal of this work was to develop intracellular one-pot scFv libraries of sufficient size and diversity to facilitate isolation of antibodies of every conceivable specificity, including antibodies with solubility, stability and affinity properties suitable/sufficient for intracellular expression. An important determinant of the composition and diversity of this *alternative* in vitro immune system is the source of antibody genes used as building blocks to construct the library. In order to explore the probability of isolating intrabodies directly from primary (mouse) antibody repertoires, we have engineered a single pot antibody library in an activation domain vector and tested it in a two-hybrid selection strategy by using a wide panel of different antigens.

The results obtained from all the different selections performed have indicated that the SPLINT library is a good source of antibody fragments, able to bind proteins intracellularly. In fact, it was possible

to select several scFv against each antigen challenged with the IAC technology. For most of the target antigens, it was possible to isolate several antibodies, thus indicating that, although this proof of principle SPLINT library has a complexity of $\sim 10^7$, the diversity obtained is still sufficient to isolate antibody binders that recognize particular antigens as, for example, proteins that are highly conserved between species.

It is very well reported that the probability to find antibodies of a given affinity in a library has a direct relationship with the size of the library itself, in such a way that higher affinity antibodies can be selected from highly diverse libraries of greater complexity (Perelson and Oster, 1979). The required diversity for the selection of high affinity antigen-specific antibodies from an immune library is much lower than that required for such selection from a non-immune library. On the other hand, the conditions of intracellular expression allow antigen–antibody pairs to achieve higher local concentrations, making affinity as would be determined in vitro not the most important parameter for being a good, functional intrabody. We have demonstrated that a relatively small library, derived from a natural repertoire, contained functional intrabodies against all antigens against which it was challenged, and can therefore provide a good source of antibodies suitable for intracellular expression.

The SPLINT library was constructed with no engineering whatsoever of the murine V genes, such

as framework consensus engineering or CDR grafting on stable frameworks.

Contrary to previous expectations, there are enough well-behaved intracellularly stable antibodies in a small naïve library of 10^7 transformants, grown in this format, to select functional binders under the relatively low affinity selection conditions which yeast two-hybrid provides. This certainly contrasts with the well-established observation (Visintin et al., 2002; Tse et al., 2002) that a very high proportion of ELISA-positive antigen-specific scFv fragments isolated from phage display libraries fail to pass the *in vivo* IACT selection against the same antigen. How can we interpret these results? First of all, it is possible (although unlikely) that phage display selects antibodies that are particularly unsuitable for intracellular expression, as opposed to antibodies that are directly derived from natural repertoires, with no intermediate selection. Second, unfolded or aggregating antibodies certainly provide a growth disadvantage to cells, and this is likely to contribute a background of antigen-independent selection that provides an enrichment for functional intrabodies in the SPLINT library, which are then selected in an antigen-dependent way. Third, the relatively lower affinity requirement for intrabodies reduce the size of the library necessary to isolate intracellular binders. Fourth, it is well established that half the antibodies expressed in human early B cell compartments are polyreactive, and a high proportion of the polyreactive antibodies are counterselected during B cell differentiation (Wardemann et al., 2003). A high frequency of polyreactive clones in the naïve antibody population used to construct the present SPLINT could in part explain the success in isolating antibodies against all antigens, although our secondary screening results (see Fig. 5) show that specific antibodies can indeed be readily found from SPLINT. Last but not least, it is possible that the effective diversity of the library is actually higher than the initial number of yeast transformants, if more than one scFv is expressed in any given cell, and this may or may not result in further recombination events and in segregation of different scFvs in daughter cells. The latter point, presently being carefully evaluated, could easily contribute to an increase of the effective diversity by a factor 10 or more, and under the selective pressure of IACT this may then lead to the selective isolation of one antigen-specific scFv fragment.

Only future experiments will address these possibilities. A larger SPLINT library could readily be accomplished either by directly scaling up the present procedure or by recombination methods as has been carried out in yeast displayed antibody libraries (Feldhaus et al., 2003). Following the proof of principle provided in this paper, the construction of a large SPLINT library from human V regions is presently under way.

IACT, in combination with SPLINT, now allows the direct selection of scFv suitable for intracellular expression obviating the need for a preliminary *in vitro* phage antibody screen with protein antigen and the need for any modification or randomization of CDRs on fixed consensus framework based on intracellular consensus sequences (ICS) (Tanaka et al., 2003; Tanaka and Rabbitts, 2003). However, if required, ICS-based engineered V regions can be constructed in the SPLINT format, and this could increase the effective diversity of the SPLINT library.

It is noteworthy that the SPLINT selection procedure overcomes the protein expression and purification bottleneck, allowing the isolation of antibodies directly from gene sequences with no protein manipulation. The application of this technology will be very powerful for the large-scale generation of scFv specific for a cDNA or EST-encoded proteins, because it will require only cloning of the sequences in the DNA-binding domain vector (Chambers and Johnston, 2003). This would be very useful especially in functional genomics and proteomics programs, where a high throughput and rapid system is required.

Larger-scale identification of antigen–antibody interactions in traditional IACT could become a laborious, time-consuming task because each positive clone must be picked out individually and the scFv DNA must be separately isolated and sequenced. However, there are now commercially available robotic two-hybrid array kits and fully automated two-hybrid systems that screen up to 100,000 clones per day. With this approach, the user needs only to sequence the clones when the array is initially constructed. Thereafter, since the same array is used repeatedly, positive transformants can be readily identified without additional sequencing and polyreactive antibodies could be removed from the screenings very easily.

This will also improve considerably the information derived from intrabody-V regions sequences, thus

further developing the Validated Intrabody Database (VIDA), which contains a large number of sequences of validated ICABs. VIDA was developed to predict whether a given antibody is a good intracellular antibody or not and is available as a resource (<http://www.laylinegenomics.com/vidamage/index/htm>). The optimization of the VIDA set will allow facilitating and accelerating our understanding of intracellular antibody properties thus allowing a rapid improvement of the related protein knockout technology.

We believe that with the development of SPLINT libraries, the use of intracellular antibodies for intra-

cellular protein knockouts will be greatly accelerated as they provide fully validated antibodies for intracellular function. This makes the intracellular antibody technology a very good complement to other interference methodology, including the now widely used RNAi methods (Martienssen, 2003; Wall and Shi, 2003; Zhang et al., 2003). However, the intracellular antibody technology for selective protein knockout permits distinctive advantages for proteomic applications.

First, targeting the protein instead of the RNA bypasses the so-called “RNA reserve problem”,

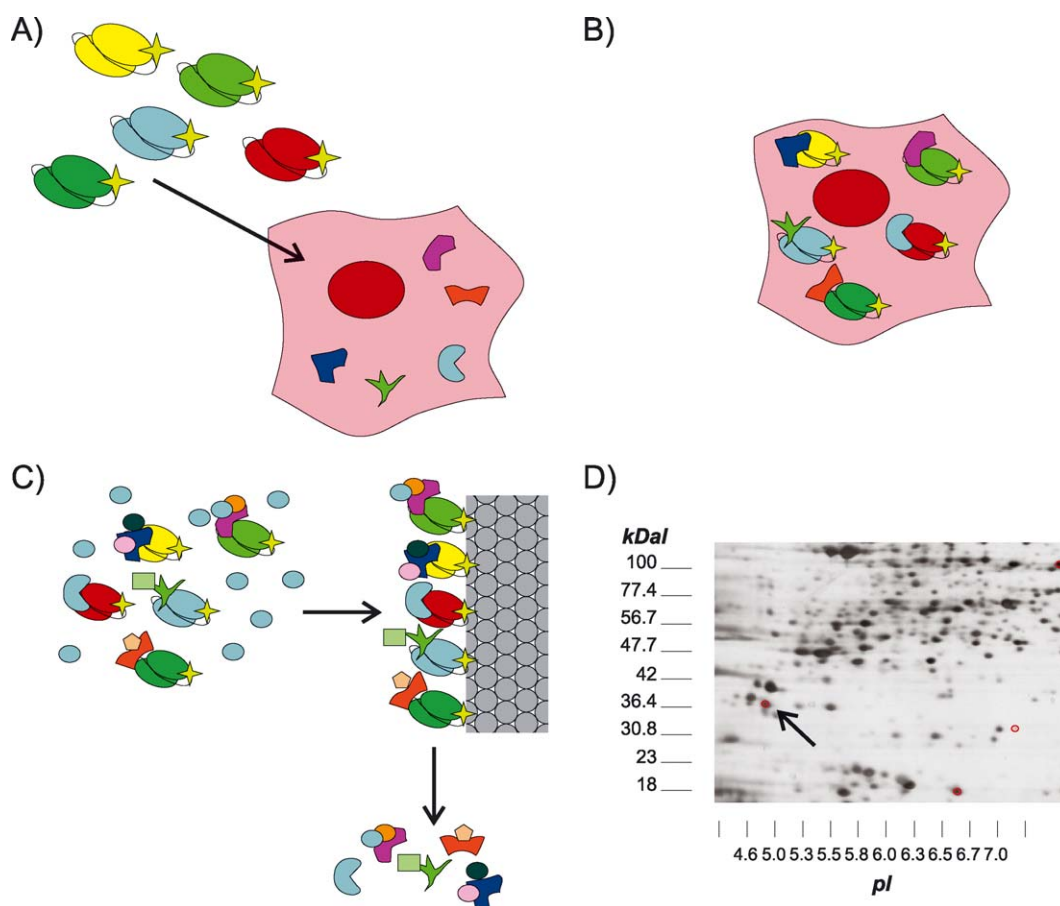


Fig. 9. Pull-down technology with intracellular antibodies. The technology could use in vivo selection of a cDNA library created from a cell line and the SPLINT antibody library. After isolation of a large panel of Ag–scFv pairs, the isolated genes could be identified and the scFv they recognize could be sequenced. (A) Isolated scFv are then expressed as a cell-specific library in vivo, in the target cell of interest and used to recapture in vivo the proteins expressed in the cell, (B) either directly or after a phenotypic selection. (C) The intracellular scFv–protein complexes could then be immunoprecipitated using the scFv sequence tag and analyzed using classic proteomic techniques such as 2Dgel and MS (D).

according to which it is not uncommon that 80–90% reduction in RNA levels still results in full protein function, due to higher protein half-life.

Second, with antibodies isolated against protein–protein interaction domains, it is possible to selectively inhibit a subset of the protein “functions” while leaving the others intact.

Third, if antibodies are used in functional selection schemes, they can be used downstream for “pull-down” and isolation of the recognized proteins that can be identified with proteomics means (Fig. 9).

In general, since proteins are much closer to biological function than the corresponding mRNA, protein knockout methodologies are more suitable to study protein function and SPLINT libraries represent an optimal source to do so.

With respect to other protein function interference methods, there are many ways in which intrabodies can be used inside the cell to affect protein function. Apart from blocking protein–protein or protein–nucleic acid interactions, it is possible to design schemes whereby the antibody binds antigen and relocates it to an inappropriate subcellular location (traffic diversion). Intrabodies can also be employed to inhibit directly the function of an enzyme or to promote the death of target cells by inducing caspase-3 mediated apoptosis (Tse and Rabbitts, 2000). The antibody-mediated selective degradation of cellular protein targets, via the ubiquitin–proteasome pathway, is also a promising approach (suicide antibodies) (Cattaneo and Biocca, 1997; Zhang et al., 2003). Harnessing the cellular machinery to inhibit intracellular protein functions represents a great upside potential of the whole intrabody approach.

We believe that the intrabody technology has now reached the point where it can be scaled up for large-scale functional proteomic projects, to derive intracellular antibodies directly from gene sequences. In particular, whole cell protein interaction maps are presently being derived and the aim of isolating antibodies directed against all protein–protein interactions of a given pathway or cell is not beyond reach. SPLINT libraries represent now an essential building block to engineer selection schemes to target the cell interactome with a complete set of neutralizing intrabodies.

4. Materials and methods

4.1. Construction of vectors

Construction was performed according to standard techniques.

4.2. Construction of pLinker220 plasmid

For construction of plasmid pLinker220, the following cloning strategy was applied. A PCR V_k and V_H cloned with 220 linker was performed by using these oligonucleotides: sense 5'-TCGATTTCACC-ATGGCGCCGAAAAAAGAGAAAAGTGGCC-CAGCCGAGCGCGCATGCCGATATTGTG-3' and antisense 5'-TTCTTGGGTGCCATGGCGGAGCTCGAAACGCTAGCTGAGGAGACGGT-GACCGTGGT-3'. The resulting PCR product was digested with *Nco*I and ligated to *Nco*I-digested vector VP16* (Hollenberg et al., 1995) to give pLinker220a. The activation domain VP16 was also reduced; pLinker220a was used as a template. VP16 was amplified by using these oligonucleotides: sense 5'-TCGAGCGGTACCGCTAGCGTTTCGAGCTCCGCCATGG-3' and antisense 5'-CGGCCAGTGAATTCTACTACCCACCGTACTCGTCAAT-3'.

The resulting VP16 PCR product was digested with *Nhe*I–*Eco*RI and was cloned in *Nhe*I–*Eco*RI-digested pLinker220a vector to give pLinker220.

4.3. Construction of pMIC-BD1 plasmid

For construction of plasmid pMIC-BD1 the DNA-binding domain *lexA*, terminator sequence from ADH1 and part of TRP1 gene was isolated from pBTM116 (Bartel et al., 1993) by digesting the vector with *Hind*III enzyme. The isolated fragment was cloned in the *Hind*III-digested pBD-GAL4 Cam vector to give pMIC-BD1.

4.4. Construction of pMIC-BD2 plasmid

In order to inactivate the nuclear localization signal (Rhee et al., 2000), a mutant of wild-type *lexA* was engineered. Wild-type *lexA* was mutated by using these primers: sense 5'-AAGATGAAAGCGTTAACGGC-

CAGGCAACAAGAGGTG-3' and antisense 5'-AAC-GACAATTGGTTTAAACTCGCTATTTTCTGGCA-ACAGTTCGACTTTATTGCCCTGTTTTCCAGG-CCCTTAACGGT-3'. The resulting PCR product was digested with *HpaI*-*PmeI* and cloned into *HpaI*-*PmeI*-digested pMIC-BD1 vector to give pMIC-BD2.

4.5. SH2-Shc/MIC-BD1

The DNA fragment SH2-Shc was PCR-amplified from pGEX4T1-p66Shc (kind gift of P.G. Pelicci) and cloned between *EcoRI*/*BamHI* restriction sites of pMIC-BD1 plasmid.

4.6. CH2-Shc/MIC-BD1

The DNA fragment CH2-Shc was PCR-amplified from pGEX4T1-p66Shc and cloned between *EcoRI*/*BamHI* restriction sites of pMIC-BD1 plasmid.

4.7. Syk/MIC-BD2

The Syk *EcoRI*/*BamHI* fragment from Syk/BTM116 (Visintin et al., 1999) was subcloned between *EcoRI*/*BamHI* restriction sites of pMIC-BD1 plasmid.

4.8. K-RAS/MIC-BD1 and K-RAS/MIC-BD2

K-RAS *BamHI*/*PstI* from K-RAS/BTM116 (Visintin et al., 1999) was subcloned between *BamHI*/*PstI* restriction sites of pMIC-BD1 and pMIC-BD2, respectively.

4.9. LT β 4/MIC-BD1

The DNA fragment LT β 4 was amplified from LT β 4/pCI-neo vector (gently provided by A. Hayday) and cloned between the *EcoRI*/*BamHI* restriction sites of pMIC-BD1 plasmid.

4.10. UT β 4/MIC-BD1

The DNA fragment LT β 4 was amplified from UT β 4/pCI-neo vector (gently provided by A. Hayday) and cloned between the *EcoRI*/*BamHI* restriction sites of pMIC-BD1 plasmid.

4.11. Gephyrin/MIC-BD1

The DNA fragment of the 153–348 domain of gephyrin protein was amplified from rat gephyrin (gently provided by H. Betz) and cloned between the *EcoRI*/*BamHI* restriction sites of pMIC-BD1 plasmid (this construct was gently provided by E. Cherubini).

4.12. A β 1–42/MIC-BD1

The DNA fragment of β amyloid 1–42 domain was synthesized by primers annealing and then cloned into *BamHI*/*PstI* restriction sites of pMIC-BD1 plasmid.

4.13. TFII-I 1–397/MIC-BD1

The DNA fragment of the 1–397 domain of TFII-I transcription factor was amplified from human TFII-I cDNA and cloned into *BamHI* restriction site of pMIC-BD1 plasmid.

4.14. X5 ORF SARS-CoV/MIC-BD1

The DNA fragment of the X5 ORF of SARS-Coronavirus was synthesized by primers annealing and then cloned into *EcoRI*/*SaII* restriction sites of pMIC-BD1 plasmid.

All clones were sequenced, using the Epicentre Sequitherm Excel II kit (Alsby, Mill Valley, CA), with a Li-Cor 4000L automatic sequencer (Lincoln, NE).

Before starting selection of SPLINT library, all baits were tested for transactivation of reporter genes as described (Visintin and Cattaneo, 2001). All baits resulted to be non-transactivating and thus used for the selection strategies.

4.15. Construction of SPLINT library

Six months mice were euthanized by cervical dislocation just prior to spleen removal. Spleens were teased apart using forceps and sterile needle to produce a single cell suspension. Lymphocytes were used immediately for RNA preparation (Qiagen). cDNA was synthesized using random hexamers and reverse transcriptase following standard protocols. V λ , V κ

and VH regions from random-primed cDNA were amplified using a modified version of primers described (Orlandi et al., 1992). VH and VL genes were re-amplified to add a region of overlap in the scFv linker as well as long tails to facilitate restriction enzyme digestion. The scFv were assembled by mixing equimolar amount of VH and VL genes and performing assembly as previously described (Kreber et al., 1997). The scFv obtained were cloned into *Bss*HII/*Nhe*I-digested pLinker220 to obtain a library of $\sim 10^7$ different clones in L40 yeast cells. SPLINT library was transformed into L40 yeast strain by using a rearranged lithium acetate transformation protocol (Visintin and Cattaneo, 2001). SPLINT library and a panel of baits were cotransformed into yeast cells as described (Visintin and Cattaneo, 2001; Visintin et al., 2002). Colonies were isolated on histidine-deficient plates and screened for β -gal activity on filters as described (Visintin and Cattaneo, 2001; Visintin et al., 2002).

4.16. Intrabody binding specificity

Isolated intrabodies were subcloned into the plasmids pDAN3 between *Bss*HII–*Nhe*I restriction sites. The intrabodies were expressed from the plasmid pDAN3 (Sblattero and Bradbury, 2000) in HB2151 *E. coli*, grown at 30 °C for 4 h after induction with 0.5 mM IPTG and 50 μ l culture supernatant containing antibody fragment were used in ELISA as described (Marks et al., 1991). Ninety-six-well plate was coated with 100 μ l/well of amyloid β 1–42 peptide and BSA as a control (10 μ g/ml in PBS). The in vitro binding specificity of all scFv was determined by ELISA using the target antigen and BSA as substrate.

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